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Intracellular localization of varicella-zoster virus ORF39 protein and its functional relationship to glycoprotein K

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Abstract

Varicella-zoster virus (VZV) encodes two multiply inserted membrane proteins, open reading frame (ORF) 39 protein (ORF39p) and glycoprotein K (gK). The HSV-1 homologs of these proteins are believed to act in conjunction with each other during viral egress and cell–cell fusion, and they directly influence each other's intracellular trafficking. However, ORF39p and VZV gK have received very limited study largely due to difficulties in producing antibodies to these highly hydrophobic proteins. To overcome this obstacle, we introduced epitope tags into both ORF39p and gK and examined their intracellular distributions in transfected and infected cells. Our data demonstrate that both ORF39p and gK accumulate predominately in the ER of cultured cells when expressed in the absence of other VZV proteins or when coexpressed in isolation from other VZV proteins. Therefore, the transport of VZV ORF39p and gK does not exhibit the functional interdependence seen in their HSV-1 homologs. However, during infection, the primary distributions of ORF39p and gK shift from the ER to the Golgi, and they are also found in the plasma membrane indicating that their intracellular trafficking during infection depends on other VZV-encoded proteins. During infection, ORF39p and gK tightly colocalize with VZV envelope glycoproteins B, E and H; however, the coexpression of ORF39p or gK with other individual viral glycoproteins is insufficient to alter the transport of either ORF39p or gK.

Keywords: Varicella–zoster virus; Open reading frame 39; Glycoprotein K; Intracellular localization

Introduction

Varicella-zoster virus (VZV) is classified as an alphaherpesvirus based largely on its growth characteristics and its ability to establish latency in the nervous system. However, unlike herpes simplex virus types 1 and 2 (HSV-1 and -2), and most other non-human alphaherpesviruses, VZV induces syncytia and is highly cell associated when grown in cultured cells indicating that its egress pathway differs substantially from that of other alphaherpesviruses (Arvin, 1996; Grose et al., 1979).

During herpesvirus egress, nucleocapsids acquire their initial envelope upon budding through the inner nuclear membrane into the perinuclear space and subsequently lose their primary envelopes as they fuse with the outer nuclear membrane to release capsids into the cytoplasm (Harson and Grose, 1995; Mettenleiter, 2002; Spear et al., 1978; Skepper et al., 2001). Cytoplasmic capsids ultimately acquire their final envelopes by budding into Golgi-derived membranous structures that, accordingly, must contain the full complement of virus-encoded membrane proteins found in the envelope of mature virions (Gershon et al., 1994; Zhu et al., 1995). Mature virions are then transported within vesicles to the surface of cells where they are released upon fusion of the transport vesicles with the plasma membrane (Johnson and Huber, 2002).

Several alphaherpesvirus envelope proteins are believed to participate in viral egress including UL20 protein (the homolog of VZV ORF39 protein) and glycoprotein K (gK). These proteins are conserved in all alphaherpesviruses, but most of what is known about them is based on the study of the HSV-1 and pseudorabies virus (PRV) homologs. In HSV-1, syncytial mutations have been mapped to both gK and UL20 protein, and HSV-1 or PRV unable to express either gK or UL20 protein are not released from the cytoplasm into the extracellular space;

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rather, they accumulate within cytoplasmic vesicles, suggesting that these proteins are required for the fusion of intracellular membranes (Baines et al., 1991; Banfield and Tufaro, 1990; Dolter et al., 1994; Fuchs et al., 1997; Mo et al., 1999). In addition, HSV-1 gK and UL20 protein appear to be functionally interdependent (Foster et al., 2004b). It has been shown, for example, that these proteins depend upon one another for their transport to post-ER compartments and that they colocalize to the *trans*-Golgi network after endocytosis from the plasma membrane. In addition, a functional link between HSV-1 gK and UL20 protein has been demonstrated during virus mediated cell–cell fusion (Avitabile et al., 2004; Foster et al., 2004a; Hutchinson et al., 1992b; Melancon et al., 2004). Based on these findings, it has been hypothesized that HSV-1 and PRV gK and UL20 protein interact directly or indirectly during cytoplasmic virion envelopment and viral egress.

Both VZV gK and ORF39 protein (ORF39p) are structurally similar to their counterparts in HSV-1 and PRV. In particular, they are highly hydrophobic and predicted to be multipass transmembrane proteins (Foster et al., 2003). While it seems likely that these proteins, like their homologs in HSV and PRV, are involved in viral egress, the amino acid sequences of both VZV ORF39p and gK diverge markedly from their HSV homologs (22% and 28% identity, respectively). This, coupled with the dissimilarity of VZV and HSV growth properties, raises the possibility that these proteins may function differently from their HSV homologs during viral egress.

In this report, we describe the intracellular localizations of VZV ORF39p and gK and examine their functional relationship to one another. We demonstrate that ORF39p and gK remain in the ER in the absence of other viral proteins, but accumulate in the Golgi during infection, indicating that they are dependent upon other VZV proteins for their intracellular trafficking. However, unlike their homologs in HSV, their intracellular transport to the Golgi is not mediated solely by their coexpression. In addition, VZV ORF39p and gK tightly colocalize with each other during infection as well as with other VZV envelope glycoproteins, suggesting a role for ORF39p and gK at the site of final envelopment.

Results

Expression and characterization of ORF39p and gK containing epitope tags

Efforts to generate both polyclonal and monoclonal antibodies to VZV ORF39p and gK have met with limited success, presumably due to the highly hydrophobic nature of these proteins (unpublished data). To date, only polyclonal antibodies to VZV gK have been produced (Mo et al., 1999), and these lack sufficient specificity to conduct detailed localization studies (Heineman and Hall, unpublished data). Therefore, epitope tags were incorporated into gK and ORF39p to facilitate their detection. In the case of gK, a FLAG epitope was inserted near its amino terminus (gK-F), but following aa 31 in order to avoid removal of the epitope by cleavage of the predicted gK signal peptide after aa 24. To provide additional flexibility in the detection of ORF39p, epitopes were inserted at both the amino and carboxyl termini of ORF39p—a FLAG epitope was inserted at its amino terminus (ORF39p-F), and a cMyc epitope linked to a polyhistidine epitope was inserted at its carboxyl terminus (ORF39p-M) (Fig. 1A).

To demonstrate that ORF39p and gK could be detected using the inserted epitopes, ORF39p-F, ORF39p-M and gK-F were expressed in cultured cells by transfection and immunoprecipitated with either anti-FLAG or anti-cMyc MAbs. Upon resolution by SDS-PAGE, ORF39p-F migrated as a 28 kDa

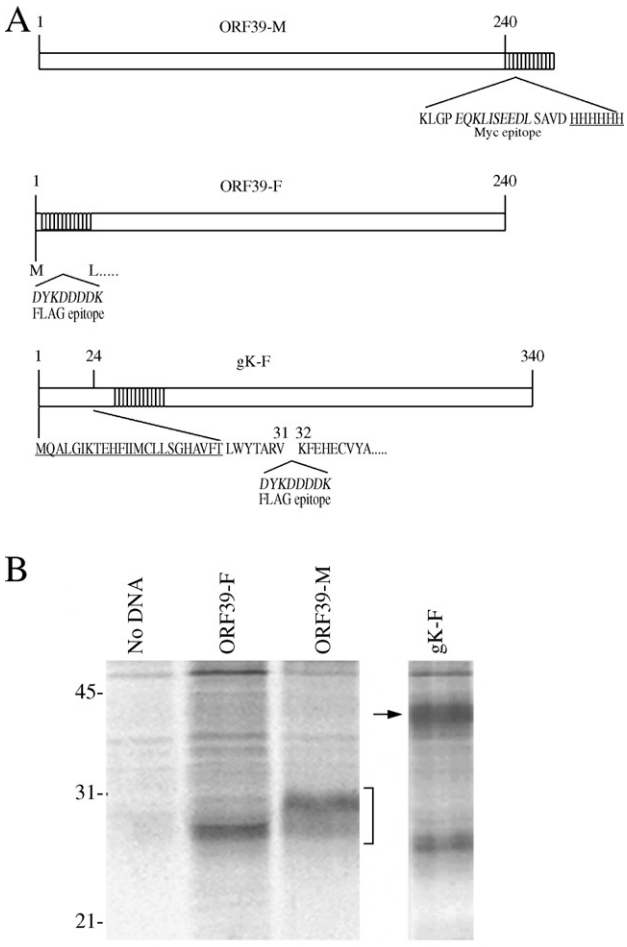

Fig. 1. Expression and detection of ORF39p and gK. (A) Schematic of constructs encoding epitope-tagged forms of ORF39p and gK. In the top two lines, the white bar represents the 240 amino acid (aa) ORF39p. To produce ORF39-M, 25 amino acids were inserted following the last native amino acid in the ORF39p (top line). These include the cMyc epitope (italics) and the polyhistidine epitope (underlined). To produce ORF39-F, the 8 aa FLAG epitope (italics) was inserted following the ORF39 start codon (second line). A non-native leucine codon (L) was incidentally inserted following the FLAG epitope. In the third line, the white bar represents gK, which contains 340 aa. The 24 aa that comprise the predicted signal sequence and the subsequent 16 aa are shown. The FLAG epitope (italics) was inserted between aa 31 and 32. (B) Immunoprecipitation of ORF39p and gK. Cells were transfected with ORF39-F, ORF39-M, gK-F or no DNA, metabolically labeled (Tran^{35}S-label) and immunoprecipitated using either anti-FLAG or anti-cMyc MAbs. ORF39-F migrates as a 28 kDa species and ORF39M migrates as a 30 kDa species (bracket), and gK-F migrates as a 39 kDa species (arrow).

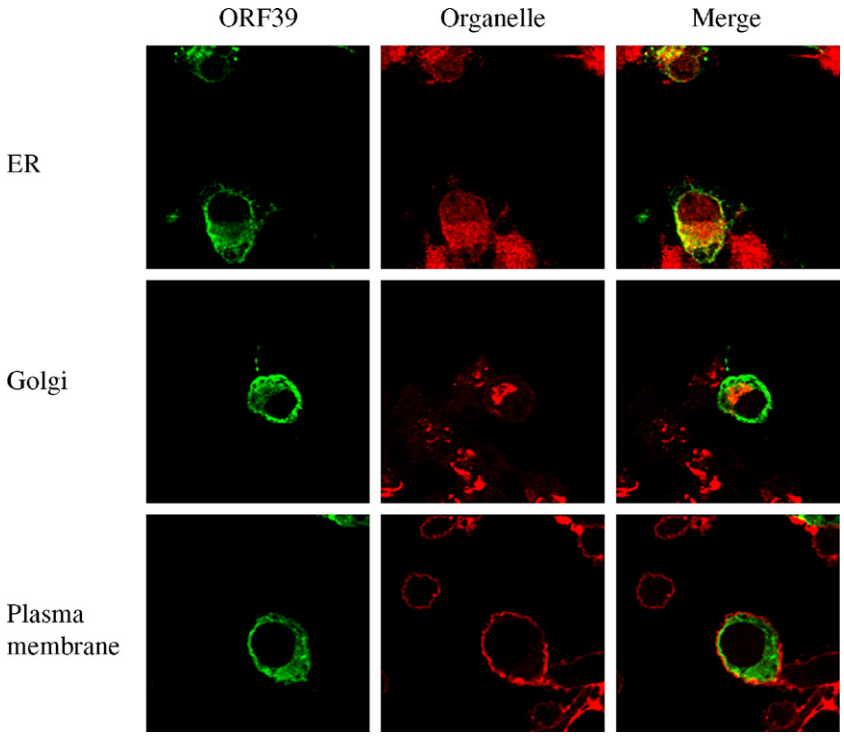

Fig. 2. Intracellular localization of transiently expressed ORF39p. Cells transfected with ORF39-F were fixed at 16 h post-transfection and incubated with anti-FLAG MAbs to visualize ORF39p (green), and specific organelle markers that recognized the ER (Grp94-C), Golgi (giantin) or plasma membrane (cell surface biotinylation) (red).

species, ORF39p-M as a 30 kDa species and gK-F as a 40 kDa species (Fig. 1B). The calculated masses of ORF39p and gK are 27.1 and 38.6 kDa, respectively (Davison and Scott, 1986). Therefore, the sizes of the epitope-tagged forms of ORF39p and gK are in close agreement with their predicated molecular masses given that the FLAG epitope is expected to add 1 kDa

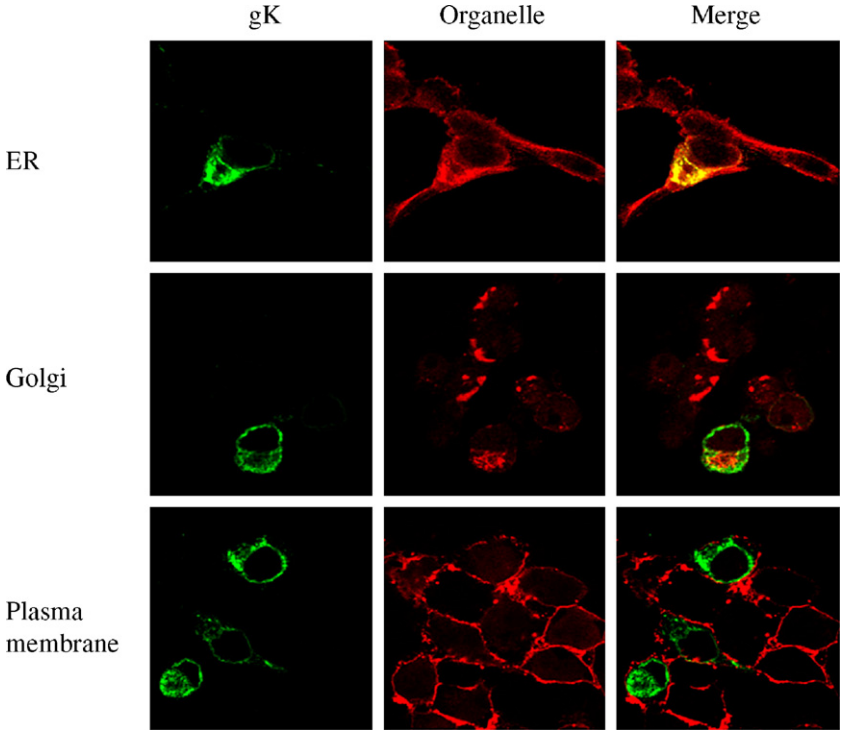

Fig. 3. Intracellular localization of transiently expressed gK. Cells transfected with gK-F were fixed at 16 h post-transfection and incubated with anti-FLAG MAbs to visualize gK (green), and specific organelle markers that recognized the ER (Grp94-C), Golgi (giantin) or plasma membrane (cell surface biotinylation) (red).

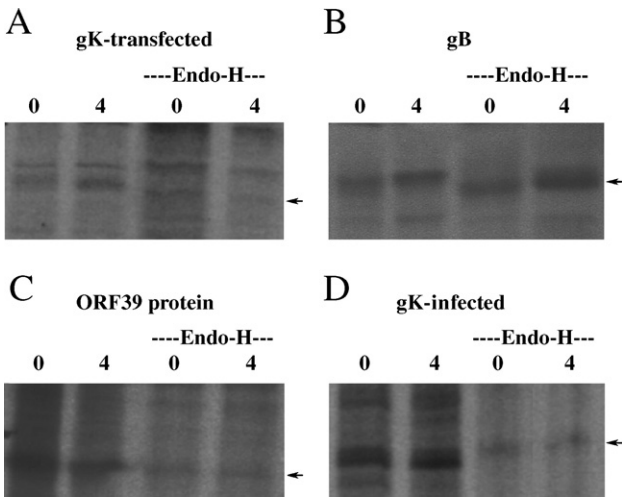

Fig. 4. Endo-H sensitivity of transiently expressed gK. Cells were transfected with plasmids encoding ORF39-F, gB or gK-F or infected with gK-F. Cells were metabolically labeled and chased in EMEM for 0 h or 4 h; these proteins were immunoprecipitated using either anti-FLAG or anti-gB MAbs. The proteins were subjected to endo-H treatment and analyzed by SDS-PAGE (arrows denote the protein of interest in each panel). (A) gK-F from transfected cells at 0 and 4 h in either the presence or absence of endo-H. (B) gB at 0 and 4 h in either the presence or absence of endo-H. (C) ORF39-F at 0 and 4 h in either the presence or absence of endo-H. (D) gK-F from infected cells at 0 and 4 h in either the presence or absence of endo-H.

and the cMyc epitope (which also contains the poly-His tag) is expected to add approximately 3 kDa. These data confirm that the inserted epitopes allow the detection of apparently full-length forms of ORF39p and gK. In addition, the ability of anti-FLAG MAbs to detect ORF39p-F, in which the FLAG epitope is at the amino terminus of ORF39p, indicates that ORF39p does not possess a cleavable N-terminal signal sequence.

Subcellular localization of ORF39p and gK in the absence of other viral proteins

In order to assess the intracellular distribution of ORF39p and gK in the absence of other VZV proteins, ORF39p-F and gK-F were transiently expressed in cultured cells by transfection. Following fixation and permeabilization, ORF39p-F and gK-F were identified by indirect immunofluorescence using anti-FLAG MAbs, and their cellular distributions were compared to those of Grp94-C and giantin, markers of the endoplasmic reticulum (ER) and the Golgi, respectively. ORF39p was diffusely distributed within the cytoplasmic compartment and largely colocalized with Grp94-C (Fig. 2). It also appeared in the nuclear envelope, but not within the nucleus itself. No colocalization with giantin was observed. VZV gK exhibited a similar intracellular distribution, again colocalizing primarily with Grp94-C but not with giantin (Fig. 3). These data indicate that in the absence of other VZV proteins both ORF39p and gK accumulate predominately in the ER.

In order to determine whether ORF39p and gK traffic to the plasma membranes of cells in the absence of other VZV proteins, a surface biotinylation-based method was employed (Foster et al., 2004b). This method was chosen because both the N- and C-termini of ORF39p are predicted to be cytoplasmic based on analogy to its HSV-1 homolog (Foster et al., 2003; Melancon et al., 2004). For purposes of comparison, the same method was applied to gK. Live cultured cells expressing ORF39p-F or gK-F were biotinylated on their surfaces. Following fixation and permeabilization, the plasma membranes of the cells were defined by incubation with streptavidin-conjugated Alexa Fluor 594, and ORF39p-F and gK-F were identified by indirect immunofluorescence using anti-FLAG antibodies. When transiently expressed by transfection, neither ORF39p-F nor gK-F colocalized with the plasma membranes of the cultured cells (Figs. 2 and 3). In addition, gK was not observed in the plasma membrane of transfected cells when indirect immunofluorescence was performed on non-permeabilized cells (data not shown). This

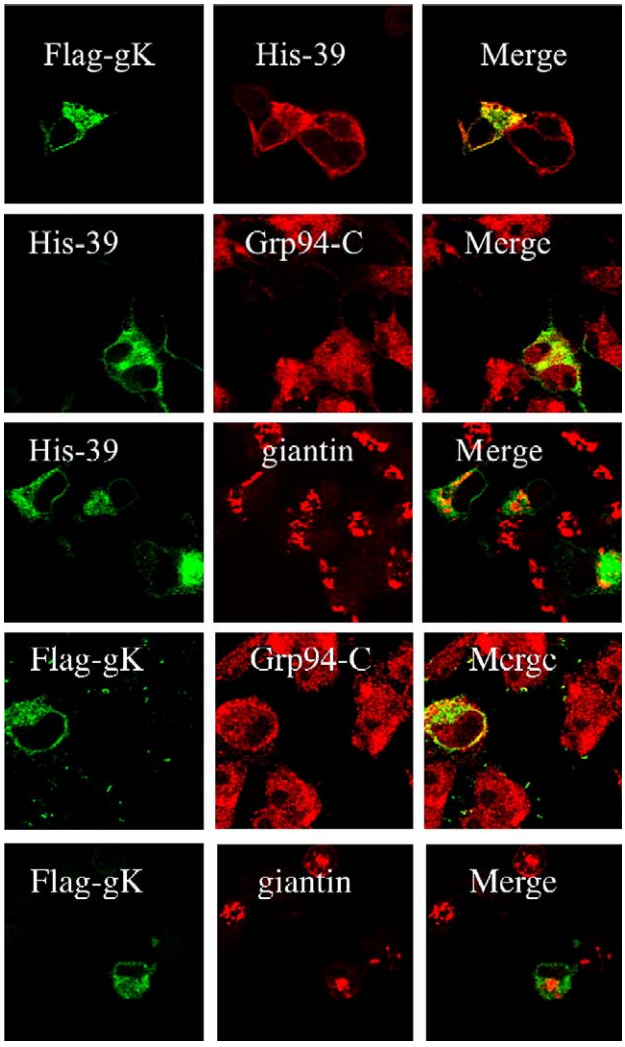

Fig. 5. Intracellular localization of transiently coexpressed gK and ORF39p. Cells were cotransfected with plasmids that express gK-F and ORF39-M. Transfected cells were fixed at 16 h after transfection and incubated with either anti-FLAG antibodies to detect gK (Flag-gK) or anti-6×-his antibodies to detect ORF39p (His-39). Transfected cells were concurrently incubated with organelle-specific markers that recognize either the ER (Grp94-C) or Golgi (giantin). For each experiment, images depicting the overlap between the signals in the first two panels are shown (merge).

supports the previous data showing that gK does not transit to the plasma membranes of cells when expressed in isolation from other VZV proteins. ORF39p was similarly not observed in the plasma membranes of non-permeabilized cells. However, this finding is not informative since both the FLAG and cMyc epitopes incorporated into ORF39p are expected to be cytoplasmic and, thus, not accessible on the surface of cells (Foster et al., 2003).

In an effort to confirm our localization data, we performed a carbohydrate analysis of gK. VZV gK has two N-linked oligosaccharides in its ectodomain that are processed in the Golgi from their high mannose (endo-H sensitive) to complex (endo-H resistant) forms (unpublished data). Thus, acquisition of endo-H resistance serves as a marker for the transport of glycoproteins from the ER to the Golgi. We found that gK expressed by transfection remained endo-H sensitive even after a 4-hour chase, indicating that it had not reached the Golgi (Fig. 4A; arrow indicates the lower MW, endo-H-cleaved form of gK). In contrast, transiently expressed VZV gB was endo-H resistant after a 4-hour chase indicating that it had been transported to the Golgi (Fig. 4B). As expected, ORF39p, which has no N-linked glycosylation sites, is unaffected by treatment with endo-H (Fig. 4C). These data, in combination with the immunofluorescence-based localization results, suggest that neither ORF39p-F nor gK are transported beyond the ER when expressed in the absence of other VZV proteins. However, gK expressed by infection exhibited endo-H resistance (Fig. 4D; arrow indicates uncleaved form of gK). Therefore, gK appears to

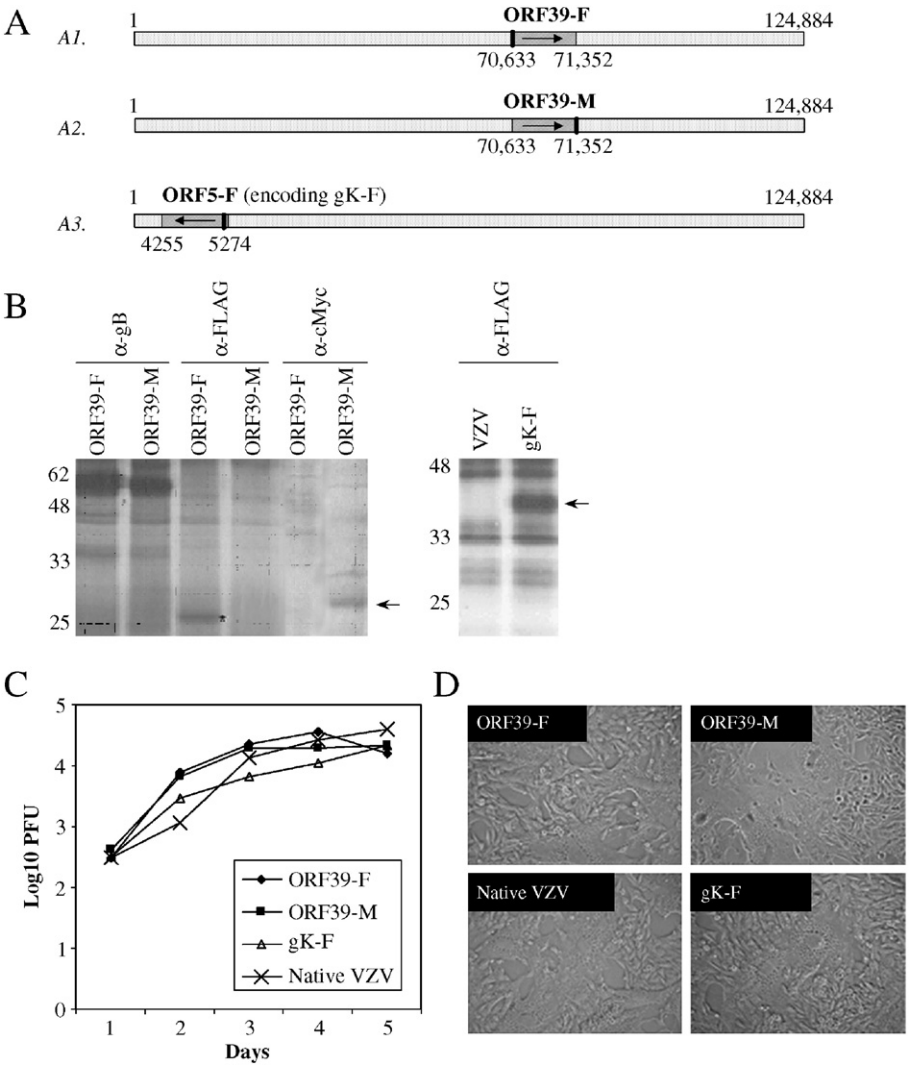

Fig. 6. Construction and characterization of recombinant viruses expressing epitope-tagged gK and ORF39p. (A) The prototype VZV genome is 124,884 bp, and the positions of ORF39 and ORF5, which encode gK, are shown (not to scale). The directions of translation are indicated (arrow). The black bars indicate the cMyc/6×-his and FLAG epitopes. (B) Cells were infected with VZV ORF39-F, VZV ORF39-M or VZV gK-F. ORF39p was immunoprecipitated with anti-FLAG (α-FLAG) or anti-cMyc antibodies (α-Myc), and gK-F was immunoprecipitated with anti-FLAG (α-FLAG) MAbs. In addition, gB was immunoprecipitated from both VZV ORF39-F and VZV ORF39-M-infected cells as an infection control. ORF39-F is denoted by the asterisk, and ORF39-M and gK-F are denoted by arrows. (C) MeWo cells were inoculated with native VZV, ORF39-F, ORF39-M or gK-F-infected cells. Cells were harvested on days 1–5 after infection, and titers were determined on MeWo cells. The $\log_{10}$ of the mean number of plaques per dish at each time point. The day 0 value is the titer of virus in the VZV-infected cell inocula. (D) MeWo cell monolayers were infected with similar titers of native VZV, ORF39-F, ORF39-M or gK-F-infected cells and incubated at 37 °C for 3 days prior to photographing.

transit to the Golgi during infection, an observation that is examined below.

Transient coexpression of ORF39p and gK does not alter the subcellular localization of either protein

Previous studies have shown that the HSV-1 UL20 protein and gK are retained in the ER when expressed alone, but transit to the Golgi and plasma membrane when expressed together (Foster et al., 2004b). To investigate if the transport of VZV gK and ORF39p shares a similar interdependence, ORF39p-M and gK-F were coexpressed in cultured cells. Upon indirect immunofluorescence, the intracellular distributions of ORF39p and gK largely overlapped (Fig. 5, top row). Similar to the pattern observed when expressed individually, both ORF39p-M and gK-F colocalized with Grp94-C, but not giantin indicating that they accumulate primarily in the ER rather than the Golgi (Fig. 5, bottom four rows). Therefore, within the sensitivity of this assay, neither ORF39p nor gK appears sufficient to influence the transport of the other protein.

Construction and characterization of VZV recombinants expressing epitope-tagged forms of ORF39p and gK

To facilitate the detection of ORF39p and gK during VZV infection, recombinant viruses that express epitope-tagged forms of these proteins were generated using cosmid-based mutagenesis (Cohen and Seidel, 1993). This method is based on the observation that transfection of susceptible cells with cosmid clones containing the entire VZV genome in overlapping fragments will recombine to produce infectious virus. To generate a VZV recombinant that expresses ORF39p containing an N-terminal FLAG epitope, the sequences encoding native ORF39p in cosmid pvPme19 were replaced with those encoding ORF39p-F to yield cosmid pvPme19-FLAG39. This cosmid was then cotransfected with the remaining three native cosmids into cultured cells. Plaques were observed 12 days following transfection indicating that infectious recombinant virus (VZV-ORF39F) had been produced (Fig. 6A1). The presence of the FLAG epitope coding sequences in frame with ORF39 in the resulting viral genome was confirmed by DNA sequencing. A similar method was used to produce a VZV recombinant expressing ORF39p containing C-terminal cMyc and poly-His epitopes (VZV-ORF39M), and the presence of the intended mutation was again confirmed by DNA sequencing (Fig. 6A2). To generate a VZV recombinant encoding gK with an N-terminal FLAG epitope (VZV-gKF), the FLAG epitope coding sequences were inserted into ORF5, which encodes gK, within cosmid pvFsp4 resulting in cosmid gK-FLAG/pvFsp4 (Fig. 6A3). These sequences were inserted into ORF5 after amino acid 31 so that they would not be removed by cleavage of the signal sequence. Transfection of gK-FLAG/pvFsp4 with the remaining three native cosmids again resulted in plaques, indicating that infectious virus had been produced. The presence of the FLAG epitope coding sequences in frame with gK in the resulting virus (VZV-gKF) was confirmed by sequence analysis. Immunoprecipitations performed on lysates from cells infected with each of the recombinant viruses (VZV-ORF39F, VZV-ORF39M or VZV-gKF) confirmed that anti-FLAG or anti-cMyc antibodies could detect the epitope-tagged forms of ORF39p and gK (Fig. 6B).

To evaluate whether insertion of the epitopes into ORF39p or gK affected the ability of VZV to replicate *in vitro*, the growth of VZV-ORF39F, VZV-ORF39M and VZV-gKF were compared to that of native VZV during a 5-day growth analysis. MeWo cells were inoculated with virus-infected cells, and daily after inoculation, the infected monolayers were harvested and the virus titers determined. The epitope-tagged recombinant viruses grew at rates similar to that of native VZV (Fig. 6C). In addition to the growth rate, plaque morphologies of the epitope-tagged viruses were compared to native VZV. Cells infected simultaneously with similar titers of native VZV, VZV-ORF39F, VZV-ORF39M or VZV-gKF were examined by light microscopy 72 h after infection. The plaque sizes and morphologies of the epitope-tagged recombinant viruses were indistinguishable from those of native VZV (Fig. 6D). Therefore, insertion of the epitopes into ORF39p and gK did not alter the growth rate of VZV or impair its ability to spread between cells.

Intracellular localization of ORF39p and gK during infection

In order to study the intracellular localization of ORF39p and gK during infection, cultured cells were infected with VZV-ORF39F or VZV-gKF. Three days after infection, the cells were fixed and permeabilized, then co-stained with anti-FLAG MAbs and polyclonal antibodies that recognize either Grp94-C or giantin, markers of the ER and Golgi, respectively. Upon

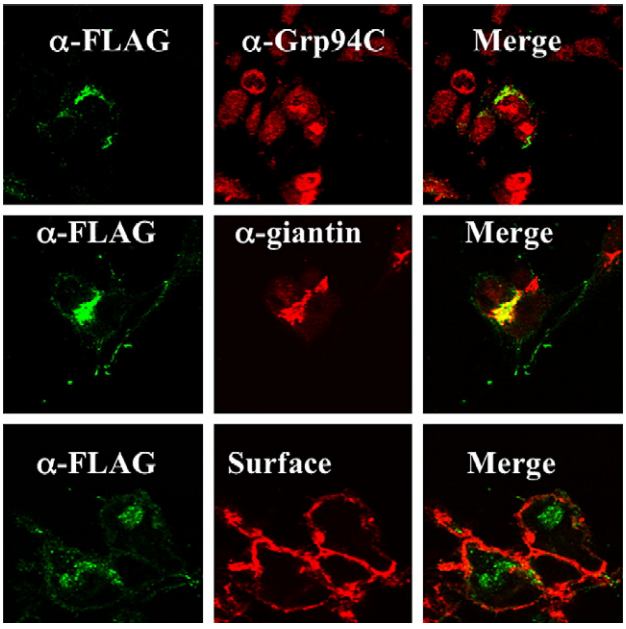

Fig. 7. Intracellular localization of ORF39p in infected cells. Cells were infected with VZV ORF39-F, fixed 72 h after infection and incubated with anti-FLAG MAbs to detect ORF39p (α-FLAG) and co-incubated with organelle-specific markers that recognized the ER (Grp94-C), Golgi (giantin) or plasma membrane (surface).

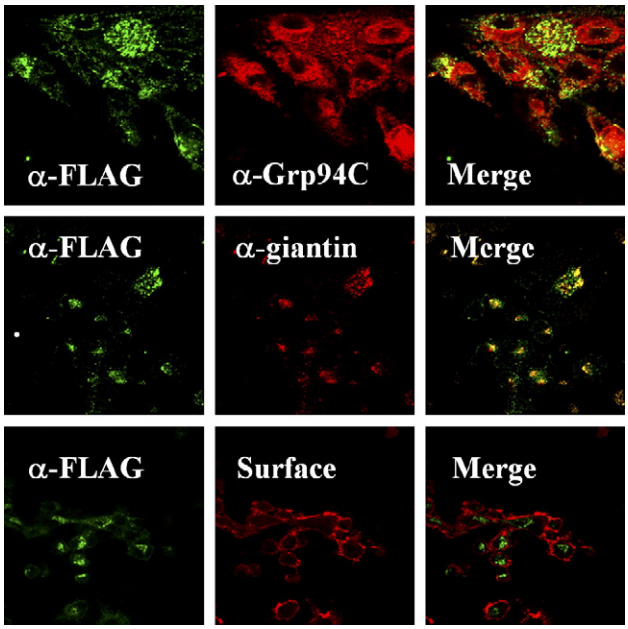

Fig. 8. Intracellular localization of gK in infected cells. Cells were infected with VZV gK-F, fixed 72 h after infection and incubated with anti-FLAG MAbs to detect gK-F (α-FLAG) and co-incubated with organelle-specific markers that recognized the ER (Grp94-C), Golgi (giantin) or plasma membrane (surface).

incubation with the appropriate secondary antibodies and examination by confocal microscopy, intracellular ORF39p and gK were seen predominately in the Golgi of infected cells based on their colocalization with giantin (Figs. 7 and 8). These results demonstrate that both ORF39p and gK traffic differently during infection than when expressed either individually or together by transfection, circumstances under which they localize primarily to the ER (Figs. 2, 3 and 5).

Colocalization of ORF39p and gK with VZV glycoproteins B, H and E

The observation that ORF39p and gK traffic differently during infection compared to transfection and that they do not appear to alter each other's transport suggests that other viral proteins may mediate their intracellular transport. As a first step in identifying possible VZV proteins that may influence the intracellular transport of ORF39p and gK, we examined whether either of these proteins colocalize with gB, gH or gE, the three most abundant VZV envelope proteins. Cultured cells infected with VZV-ORF39F or VZV-gKF were fixed and permeabilized 3 days post-infection then co-stained with anti-FLAG polyclonal antibodies and MAbs that recognize VZV gB, gH or gE. Upon incubation with the appropriate secondary antibodies and examination by confocal microscopy, both ORF39p and gK colocalize with each of the glycoproteins tested, and all appear to reside in a subcellular structure consistent with the Golgi apparatus (Fig. 9).

The observations that ORF39p and gK both colocalize with gB, gH and gE during infection and that ORF39p and gK are not sufficient to alter each other's localization in transient expression assays raise the possibility that gB, gH or gE facilitate the export of gK and ORF39p from the ER. To test this possibility, ORF39p-F and gK-F were coexpressed individually with gB or gE. When expressed with gB or gE, both ORF39p

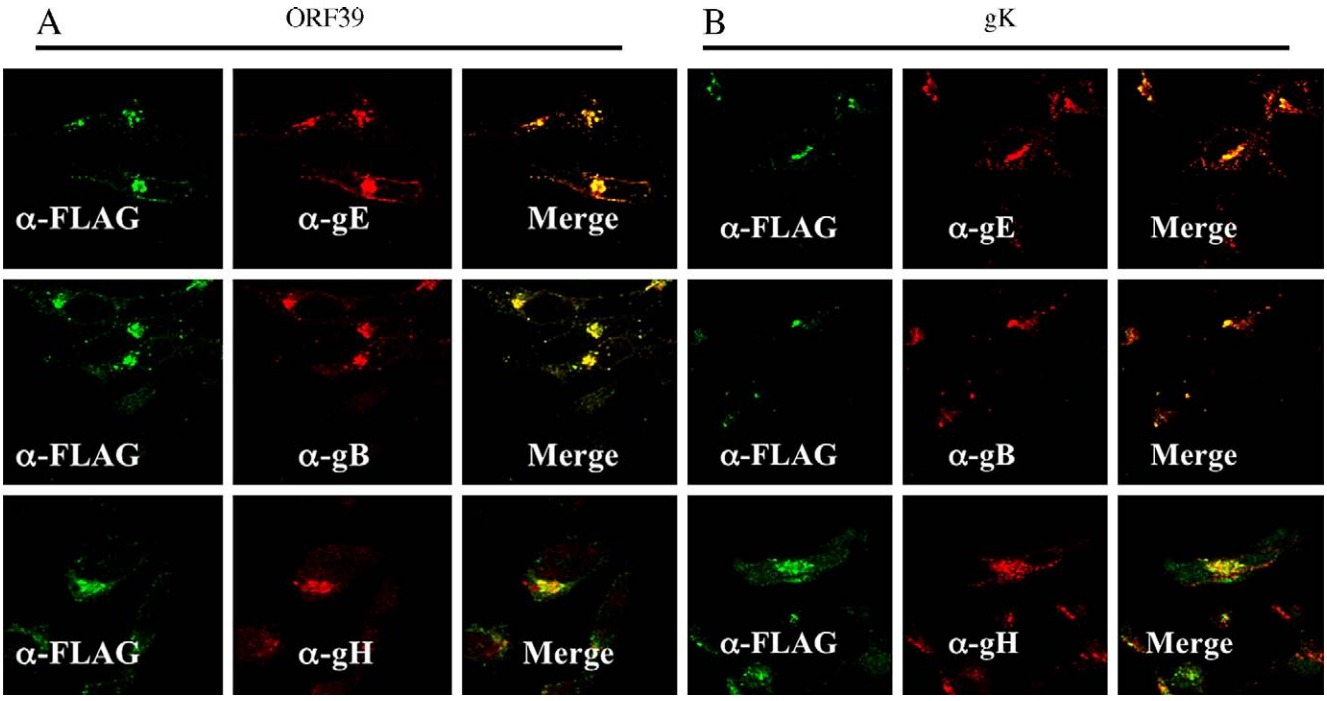

Fig. 9. Colocalization of ORF39p and gK with other VZV glycoproteins in infected cells. (A) Cells infected with VZV ORF39-F were fixed 72 h after infection and stained with anti-FLAG antibodies (α-FLAG) or antibodies specific for selected VZV glycoproteins (α-gE, α-gB and α-gH). (B) Cells infected with VZV gK-F were processed as in A. For each experiment, images depicting the overlap between the signals in the first two panels are shown (merge).

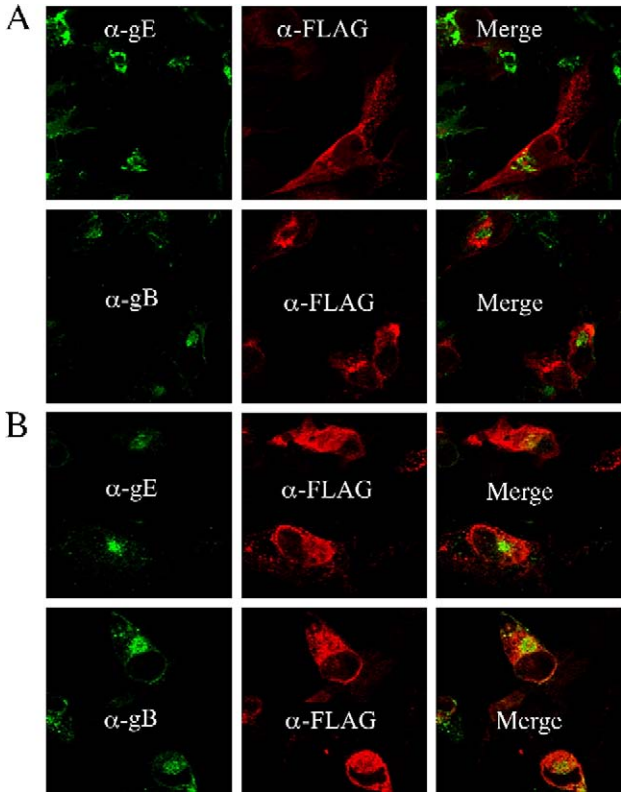

Fig. 10. Coexpression of ORF39p or gK with other VZV glycoproteins. (A) Cells were transfected with plasmids expressing ORF39-F and either gE (first row) or gB (second row). (B) Cells were transfected with plasmids expressing gK-F and either gE (third row) or gB (fourth row). For all experiments, transfected cells were fixed at 16 h after transfection and incubated with either anti-FLAG antibodies (α-FLAG) to detect either ORF39p (A) or gK (B). The cells were concurrently incubated with anti-gB (α-gB) or anti-gE (α-gE) MAbs. For each experiment, images depicting the overlap between the signals in the first two panels are shown (merge).

and gK remained predominately in the ER, demonstrating that gB or gE alone are insufficient to direct ORF39p or gK to the Golgi (Fig. 10).

Discussion

In this study, we examined the intracellular transport of VZV ORF39p and gK. We found that both ORF39p and gK accumulate primarily in the Golgi and to a lesser extent the plasma membrane of infected cells. When expressed individually, however, they fail to translocate beyond the ER, indicating that one or more, as yet undefined, VZV proteins are required for their intracellular transport.

Both ORF39p and gK are conserved among the alphaherpesviruses (Cohen and Straus, 1996; Davison and Scott, 1986; Roizman, 1990). It has previously been shown that the transport of HSV-1 gK and UL20 protein (the homolog of VZV ORF39p) beyond the ER depends on the expression of both proteins. In particular, the HSV UL20 protein is necessary for the Golgi-dependent glycosylation and cell surface expression of gK (Avitabile et al., 1994; Dietz et al., 2000; Foster et al., 2004b). In contrast, however, VZV ORF39p and gK remain in the ER even when these proteins are coexpressed. Thus, our data failed to demonstrate a similar interdependence of VZV ORF39p and gK for their intracellular transport as had been seen in HSV-1. This suggests that other VZV proteins mediate the intracellular trafficking of ORF39p and gK, leading ultimately to their distribution in the Golgi and plasma membranes of infected cells.

The biological importance of this functional divergence between VZV and HSV-1 is not known. Deletions in either UL20 protein or gK in both HSV and PrV result in the accumulation of nonenveloped capsids within the cytoplasm of infected cells, indicating that the proteins function during the cytoplasmic stages of virion envelopment. They may, for example, play a role in either secondary envelopment of cytoplasmic capsids or the prevention of aberrant de-envelopment from cytoplasmic vesicles (Dolter et al., 1994; Foster and Kousoulas, 1999; Fuchs et al., 1997; Jayachandra et al., 1997; Mo et al., 1999; Ward et al., 1994). In addition, UL20 protein is necessary for virus-induced cell fusion caused by syncytial mutations in either gB or gK (Avitabile et al., 2004). All of these data suggest that UL20 protein and gK play important roles in regulating membrane fusion associated with viral egress and virus-induced cell fusion. The extreme cell association of VZV highlights differences between its egress pathway and that of most other alphaherpesviruses, which release abundant infectious cell free virions. It is tempting to speculate that functional dissimilarities between these proteins and their HSV-1 homologs may play a role in the distinct VZV egress pathway.

The inability of ORF39p and gK to escape the ER without the intervention of other VZV proteins is in itself somewhat unexpected since neither protein possesses known ER retention signals. Moreover, both contain YXXϕ motifs that would be expected to direct their accumulation in the Golgi. The membrane topology of neither VZV gK nor ORF39p is known. However, if their topologies are analogous to those of their HSV-1 homologs, these YXXϕ motifs would be expected to be exposed to the cytoplasm where they would be available to interact with the cellular transport machinery. Nonetheless, the localization of VZV ORF39p and gK to the Golgi of infected cells has implications for the functions of these proteins. Perhaps most importantly, since VZV is believed to acquire its final envelope from Golgi-derived vesicles, the accumulation of ORF39p and gK in the Golgi raises the possibility that these proteins are incorporated into the virion envelope along with gB, gH and gE. HSV-1 gK and UL20 have been shown to be present in the virion envelope (Foster and Kousoulas, 1999; Foster et al., 2001, 2003).

For a number of reasons, other VZV encoded glycoproteins seemed to be likely candidates as mediators of ORF39p and gK transport. First, all alphaherpesvirus glycoproteins are transported to the Golgi and the plasma membrane (Mettenleiter, 2002; Roizman, 1990; Ward et al., 1994). Second, examples exist of VZV glycoproteins whose transport is influenced by direct interactions with other glycoproteins, such as gH/gL and gE/gI (Alconada et al., 1998; Cai et al., 1988; Claesson-Welsh and Spear, 1986; Hutchinson et al., 1992a; Kari et al., 1992; Klupp et al., 1994; Montalvo and Grose, 1987; Whealy et al., 1990, 1992; Whitbeck et al., 1996). Third, at least one VZV

glycoprotein, gB, contains an active ER export signal (Heineman and Hall, 2002). As an initial step in testing this hypothesis, we showed that both ORF39p and gK tightly colocalize with several other abundantly expressed VZV glycoproteins, namely gB, gH and gE, in infected cells. Additional coexpression experiments, however, showed that gB and gE had no effect on the intracellular localization of VZV ORF39p or gK. Thus, the identity of the VZV protein(s) that determine the infected cell localization of VZV ORF39p and gK remains unknown. It is, of course, possible that one or more of the membrane proteins already tested participate in the intracellular trafficking of ORF39p or gK but that this effect was not observed because additional proteins are also required.

ORF39p and gK are both highly hydrophobic, and studies of these proteins have been hampered by the inability to produce high quality antibodies presumably for this reason. In this study, we have generated both amino and carboxy terminal epitope-tagged forms of ORF39p allowing flexibility to study the proteins. The ability to detect ORF39-F indicates that ORF39 does not possess a cleavable N-terminal signal sequence, suggesting that it is not a type I membrane protein. The data provided here demonstrate the utility of epitope-tagged viruses to study VZV gK and ORF39p. In neither case did the addition of an epitope tag noticeably affect the growth rate or morphology of the mutant viruses. The epitope-tagged forms of these viruses will allow future studies including more defined examinations of the topology of ORF39p and gK and an examination of their interactions with other VZV or cellular proteins.

Materials and methods

Cell culture and virus propagation

MeWo cells, an immortalized human melanoma cell line, were grown in Eagle's minimum essential medium (EMEM) containing 10% fetal bovine serum (FBS; Bio-Whittaker) and GASP (2 mM L-glutamine, chlorotetracycline, penicillin and streptomycin; Quality Biological). BSC-40, an African green monkey kidney cell line (ATCC), were grown in EMEM containing 10% FBS and GASP. Recombinant vaccinia virus vTF7-3 was obtained from the ATCC, and viral stocks were prepared and titered in BSC-40 cells. VZV was propagated in MeWo cells.

Immune reagents and intracellular markers

Anti-FLAG M2 monoclonal antibodies (MAbs) and anti-FLAG M2 MAbs conjugated to fluorescein isothiocyanate (FITC) were purchased from Sigma. Anti-polyhistidine MAbs were purchased from R&D Systems. Anti-cMyc and anti-giantin polyclonal antibodies (PAbs) were purchased from Covance Research Products. Anti-Grp94-C PAbs were provided by Dr. Michael Green (Saint Louis University). Tetramethylrhodamine isothiocyanate (TRITC) conjugated to wheat germ agglutinin (WGA) was purchased from EY Laboratories. Goat anti-mouse and anti-rabbit IgG MAbs conjugated to FITC were purchased from Sigma. Goat anti-mouse IgG MAbs Abs conjugated with Alexa Fluor 488, goat anti-rabbit IgG PAbs conjugated with Alexa Fluor 568 and streptavidin-conjugated with Alexa Fluor 594 were purchased from Molecular Probes. Anti-VZV gB and gE MAbs were purchased from Biodesign International. EZ-Link Sulfo-NHS-LC-Biotin was purchased from Pierce Chemicals.

Plasmid construction

To generate an expression plasmid that encodes ORF39 with a FLAG epitope at its amino terminus, VZV ORF39 was amplified by PCR and inserted into pFLAG-CMV2 (Kodak) downstream of, and in frame with, the FLAG epitope (MDYKDDDDK) coding sequence present within this vector to yield pFLAG-CMV2-ORF39. The sequences encoding ORF39p/FLAG were amplified by PCR of pFLAG-CMV2-ORF39 and inserted into pBluescript II-SK+ (Stratagene) downstream of the T7 promoter to yield pBS-39FLAG. To generate an expression plasmid that encodes ORF39 with a cMyc epitope at its carboxy terminus, VZV ORF39 was amplified by PCR and inserted into pCDNA3.1A (Invitrogen) upstream of, and in frame with, the cMyc (EQKLISEEDL) and polyhistidine (HHHHHH) epitope coding sequences present within this vector to yield pCDNA-ORF39Myc. To generate an expression plasmid that encodes gK with a FLAG epitope near its carboxy terminus, VZV ORF5, which encodes gK, was amplified by PCR and inserted into pBluescript II-SK downstream of the T7 promoter to yield pBS-gK, and the FLAG epitope-coding sequence was introduced into this plasmid by site-directed mutagenesis after the codon for gK amino acid 31 to yield pBS-gKFLAG.

Production of recombinant VZV

Recombinant virus was generated using four overlapping cosmid vectors that span the VZV genome: pvFsp4 (nucleotides 1–33,211), pvSpe5 (nucleotides 21,875–62,008), pvPme19 (nucleotides 53,877–96,188) and pvSpe21 (nucleotides 94,208–124,884) (Kemble et al., 2000). To produce cosmid pvPme19-FLAG39 that encodes ORF39p containing a FLAG epitope at its N-terminus, pvPme19 was cut with *Not*I and *Avr*II, and the resulting 9433 bp fragment (VZV nucleotides 62,860–72,293), which contains ORF39 (VZV nucleotides 70,633–71,352), was isolated. This fragment was cloned into pBluescript II-KS+ (Stratagene) in which the *Sma*I site had been replaced by an *Avr*II site and the *Eco*RV site had been eliminated, to yield pBSAvrΔ*Eco*RV-9433. A 2886 bp *Eco*RV/*Xba*I fragment containing ORF39 was excised from this plasmid and cloned into pBluescript II-SK+ to yield pBS-2886. ORF39 was removed from pBS-2886, and the ORF39FLAG coding sequences from pFLAG-CMV2-ORF39 were inserted in place of native ORF39 to yield pBS-2886ORF39FLAG. VZV sequences containing ORF39-FLAG were subsequently excised from pBS-2886ORF39FLAG and cloned into pBSAvrΔ*Eco*RV-9433 from which the corresponding VZV sequences had been removed. The *Not*I/

*Avr*II fragment from this construct was inserted into pvPme19 to yield pvPme19-FLAG39.

A similar cloning strategy was used to produce cosmid pvPme19-Myc39. Using pBS-2886 as the template, ORF39 was amplified by PCR with *Nhe*I and *Hin*dIII ends, and the resulting 792 bp fragment was cloned into the *Nhe*I and *Hin*dIII sites of pCDNA3.1 (Invitrogen) to introduce cMyc and 6×-his epitope coding sequences in frame at the 3′ end of ORF39 (pCDNA-ORF39). ORF39 incorporating the cMyc and 6×-his epitope coding sequences was substituted into the VZV cosmid pvPme19 using the above cloning strategy to yield cosmid pvPme19-Myc39.

ORF5, which codes for gK, spans nucleotides 5274–4252 and is contained within VZV cosmid pvFsp4. A 3919 bp fragment (nucleotides 8205–4286) that contains all but the 3′ terminal 34 bp of ORF5 was excised from pvFsp4 by *Hin*dIII digestion. This fragment was cloned into pBluescript II-KS+ to yield pBS-gK3919. The FLAG epitope coding sequences, as well as an *Xho*I site for screening purposes, were introduced into the resulting construct after gK codon 31 by site-directed mutagenesis. ORF5 containing the FLAG epitope coding sequences was then excised by digestion with *Hin*dIII and reintroduced into pvFsp4 in place of native ORF5 to yield cosmid pvFsp4/ORF5-FLAG.

To produce recombinant virus, the method of Mallory et al. (1997) was used Briefly, 60-mm-diameter dishes were seeded with 1×10^6 MeWo cells 1 day prior to transfection. Transfections were performed using calcium phosphate precipitates (CalPhos Mammalian Transfection Kit; Clontech). The cosmids were linearized with *Asc*I, and the enzyme heat inactivated, prior to transfection. Each transfection mixture contained 1.5 μg of pvSpe21 and 3 μg of each of the remaining cosmids. Five days after transfection, the cells were seeded into 75-cm^2 flasks and monitored for cytopathic effects (CPE).

Growth characteristics of VZV

VZV titers were determined by inoculating MeWo cells with serial 10-fold dilutions of virus-infected cells. VZV growth curves were generated by inoculating MeWo cells with approximately 100 plaque-forming units (pfu) of infected cells per 25 cm^2 flask (day 0). The cells in individual flasks were treated with trypsin, and the virus titer determined at days 1 to 5 after infection (Cohen and Seidel, 1993; Heineman and Hall, 2002).

To compare plaque morphologies, cultured cells were inoculated with similar titers of cells infected with either native VZV or VZV expressing the epitope-tagged forms of ORF39p or gK, incubated at 37 °C for 4 days and then photographed through an inverted light microscope.

Expression of ORF39p and gK by transfection and infection

ORF39p and gK were expressed *in vitro* using the vaccinia T7 expression system (Fuerst et al., 1986). Cells at 75–90% confluence were infected with recombinant vaccinia virus vTF7-3 at a multiplicity of infection of 10 then transfected with 4 μg of purified DNA (Qiagen) using 10 μl of Lipofectamine 2000 (Invitrogen) in 10 cm^2 wells or 2 μg of DNA and 5 μl of Lipofectamine 2000 in 1.2 cm^2 wells. Cells were incubated at 37 °C and 5% CO_2 for 16 h prior to metabolic labeling or fluorescent staining. To express ORF39p and gK during infection, cultured cells were inoculated with VZV-infected cells and incubated at 37 °C in 5% CO_2 prior to processing.

Radiolabeling and immunoprecipitation

Metabolic radiolabeling and immunoprecipitations were performed as previously described (Heineman and Hall, 2002). Briefly, ORF39p or gK expressed in cultured cells by transfection was metabolically labeled 16 h after transfection, and VZV-infected cells were labeled when extensive 2–3+ CPE was observed, typically 3–4 days following infection. Cells were incubated for 1 h in EMEM lacking cysteine and methionine and then incubated in Cys- and Met-free EMEM containing 125 μCi of Tran^{35}S-label (ICN) per ml for 4 h. Labeled cells were washed and lysed in phosphate-buffered saline (PBS) containing 1% Triton X-100, 0.5% deoxycholate and 0.1% sodium dodecyl sulfate (SDS). VZV gK and ORF39p were immunoprecipitated by incubating the labeled cell lysates with anti-FLAG or anti-cMyc MAbs (1:750 dilution) overnight at 4 °C followed by incubation with *Staphylococcus* protein G–sepharose (Pharmacia-Biotech) for 1 h at 4 °C. After washing, immunoprecipitated proteins were eluted in sample buffer containing 2% SDS and 40 mM dithiothreitol (DTT) and resolved by SDS-polyacrylamide gel electrophoresis (PAGE). The immunoprecipitated proteins were visualized by autoradiography.

Carbohydrate analysis

VZV ORF39p, gK or gB was expressed by transfection or infection and metabolically radiolabeled as described above except that the cells were incubated for 1 h in EMEM lacking cysteine and methionine prior to the addition of Tran^{35}S-label. They were incubated with the labeling medium for 1 h. At that time, they were either lysed immediately (0 h chase) or they were incubated with chase medium (EMEM containing 10% FBS, 24 μg/ml cysteine and 15 μg/ml methionine) for 4 h prior to lysis (4 h chase). ORF39p, gK or gB was immunoprecipitated using anti-FLAG or anti-gB MAbs as described above. In some cases, the immunoprecipitated proteins were treated with endoglycosidase H (endo-H). To do so, *Staphylococcus* protein G–sepharose-bound proteins were heated to 98 °C for 3 min in 10 μl of 0.2% SDS-50 mM Tris–HCl (pH 6.8). After cooling to room temperature, 10 μl of 0.15 M sodium citrate (pH 5.3) and 1 μl of endo-H (Roche) were added, and the reaction mixtures were incubated overnight at 37 °C. Sample buffer was added to the immunoprecipitated proteins, and they were resolved by 10% SDS-PAGE.

Immunofluorescence and confocal microscopy

To evaluate the intracellular localization of ORF39p and gK, infected or transfected cells were fixed and permeabilized in 4%

paraformaldehyde/0.2% Triton X-100 for 1 h at 4 °C. Following fixation, cells were incubated with Image-it signal enhancer (Molecular Probes) for 15 min at room temperature and then incubated for 1 h with the appropriate primary antibodies. The cells were then washed in PBS and incubated for 1 h at RT with secondary antibodies conjugated to a fluorescent marker. In some colocalization experiments, WGA-TRITC (5 μg/ml in PBS) was incubated with the fixed cells for 20 min concurrently with the secondary antibodies. After washing in PBS, the cells were viewed with a Bio-Rad MRC1024 scanning confocal microscope.

To evaluate the plasma membrane localization of ORF39p and gK, infected or transfected cells were washed with PBS and incubated for 30 min at RT in 10 mM EZ-Link Sulfo-NHS-LC-Biotin (Pierce Chemicals) prior to fixation and permeabilization in 4% paraformaldehyde/0.2% Triton X-100 as above. The biotinylated cells were incubated with streptavidin-conjugated Alexa Fluor 594 (1:1000 in PBS) for 1 h at RT. Immunofluorescence was visualized using a Bio-Rad 1024 laser confocal microscope.

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